Imaging of Triglycerides in Human Coronary Plaques by Color Fluorescent Angioscopy and Microscopy

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Native triglycerides (TG) deposited in the human vascular wall is not measurable or visible in vivo to date. We discovered that by exciting fluorescence at 345 nm and emitting at 420 nm, 3-amino-4-hydroxy-5-nitrobenzene sulfonic acid monohydrate (3-ANA) elicits a brown fluorescence that is characteristic of just TG. Therefore, localization of TG in coronary plaques and normal segments that were obtained from 19 human autopsy cases was examined by color fluorescent angioscopy (CFA) and microscopy using 3-ANA as a biomarker of TG. By CFA, the percentage (%) incidence of TG in 23 normal segments, 13 white plaques without lipid deposition, 18 white plaques (growth stage) with lipid deposition, 11 yellow plaques without necrotic core (mature stage), and 12 yellow plaques with necrotic core (advanced mature stage) was 95, 92, 50, 27, and 25, respectively. By color fluorescent microscopy, TG deposited mostly in the fibrotic area of the plaques. Contrary to the general belief that TG amount increases with plaque maturation, the results indicated that TG was deposited in most of the normal coronary segments, but the amount decreased with plaque maturation. If 3-ANA becomes applicable clinically, the CFA system could be used for imaging TG within coronary plaques in patients in vivo. **(Am J Cardiol 2016;118:1306−1310)**

During our search for a biomarker for TG, we discovered that it exhibited a brown fluorescence in the presence of 3-amino-4-hydroxy-5-nitrobenzene sulfonic acid monohydrate (3-ANA)[1] by excitation at 340 nm and emission at 420 nm. The present study using color fluorescent angioscopy (CFA) and color fluorescent microscopy (CFM) aimed to clarify whether the brown fluorescence excited by 3-ANA is specific for TG and, thus, show TG deposited in human coronary plaques in anticipation of clinical application.

Methods

TG singularly does not exhibit autofluorescence but when mixed with 3-ANA, brown fluorescence is evoked. Therefore, 3-ANA was used as an indicator of TG in this study. To clarify whether the fluorescent color of TG evoked by 3-ANA is characteristic, the color fluorescence of the major substances that constitute atherosclerotic plaques[2] was examined by CFM. Chemically pure substances were used for this purpose. A CFM system with a band pass filter of 340 ± 15 nm and a band absorption filter of 420 nm was used for fluorescent imaging. The details of CFM are described elsewhere.[3,4]

CFA system is consisted of a fluorescence-excitation unit, an angioscope (VecMover; Clinical Supply Co, Gifu, Japan), a fluorescence-emission unit, and a camera. The system enables observation of a coronary segment up to 7 cm in length by a single saline flush and has been approved for clinical use by the Japanese Ministry of Health and Labor, supported by National Insurance, on a commercial basis in Japan.[5] The details of this CFA system are described elsewhere.[5,6] The intensity of the fluorescence images was arbitrarily defined as strong, weak, and absent when the exposure time required for imaging was $1\geq$, $1<$ and $5\geq$, and $5s<$, respectively.[6]

The limitation of sensitivity of the CFA system was examined using the major substances comprising atherosclerotic plaques[6] as the target. It was revealed that their fluorescence was not detectable by the CFA system when their concentration was $\leq 10^{-6}$ M.[6]

The conventional angioscopy system is consisted of a 4.5-F angioscope, light source, and a 3-coupled chilled device digital camera (Olympus). The details of the procedure are described elsewhere.[5,7] Plaque by conventional angioscopy was defined as a nonmotile and protruding or lining mass clearly demarcated from the adjacent normal wall and whose shape, location, and color did not alter after saline solution flush. Plaques were further classified as white or yellow based on their surface color. A normal segment was defined as a milky white and smooth-surfaced portion of the vessel without any protrusion.[8] Surface color of the plaques was measured by an AquaCosmos image analyzer (C7746; Hamamatsu Photonics, Hamamatsu, Japan).[7]

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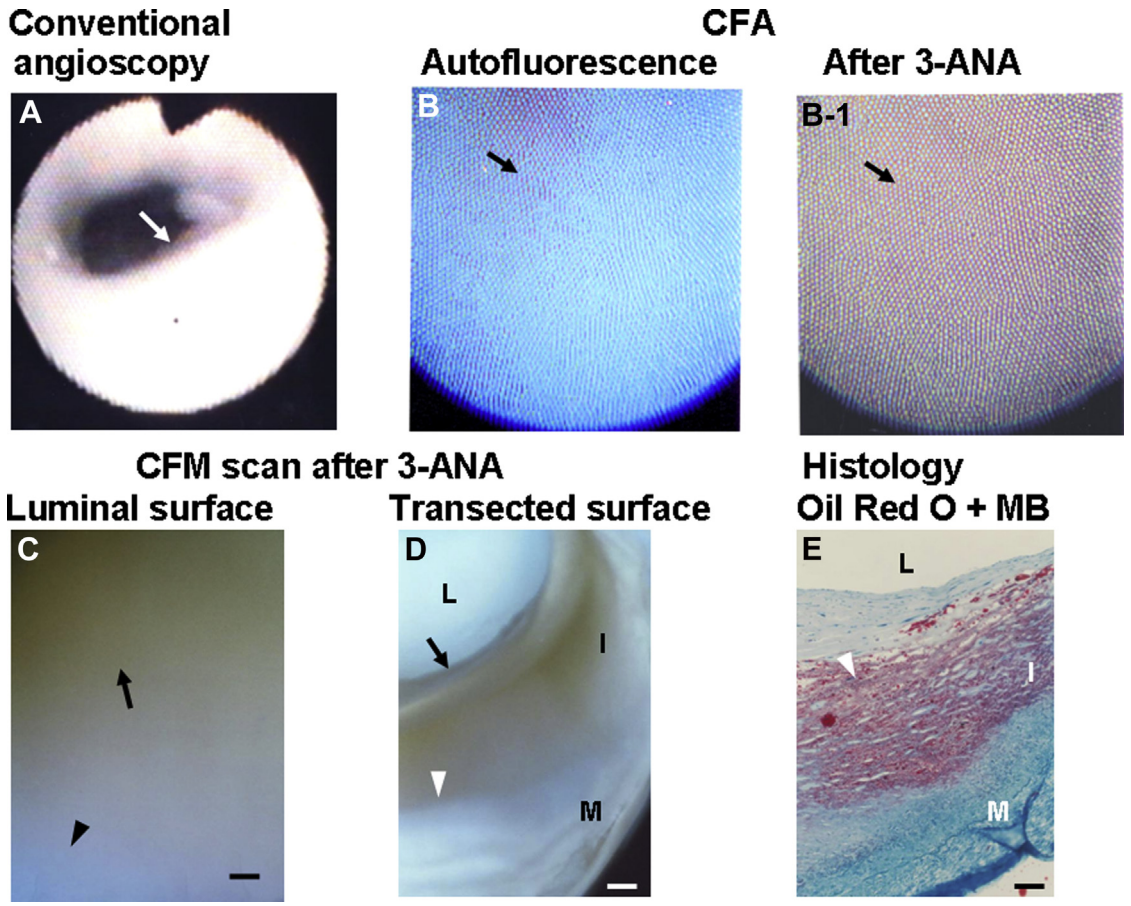

Figure 1. Color fluorescent angioscopic images of TG in a white plaque in a patient with chronic renal disease who died of acute pneumonia. *(A)* White plaque by conventional angioscopy *(arrow)*. *(B)* Blue fluorescence of the plaque was imaged by CFA before, indicating the rich collagen fibers. *(B-1)* Brown fluorescence elicited by application of 3-ANA to the same plaque *(arrow)*. *(C)* Luminal surface scan by CFM showed brown fluorescence indicating the presence of TG *(arrow)*. Transected surface of the same plaque showed deposition of TG in the inner portions of the plaque where lipid deposition was not observed *(arrow in D)*. Deposition of TG was not observed in the outer layer where lipids deposited *(arrowheads in D and E)*. *(L, I, and M)*: Lumen, intima, and media, respectively. Scale bars = 100 μm.

This in vitro study was carried out with the approval of the Ethical Committees of Japan Foundation for Cardiovascular Research, Funabashi-Futawa Hospital and Toho University. After obtaining the written informed consent of the families concerned, 29 coronary arteries (17 left anterior descending arteries, 5 left circumflex arteries, and 7 right coronary arteries) were excised from 19 successive autopsy cases from April 1, 2008, to November 1, 2014 (age 61 ± 3 years, 7 women and 12 men): death from acute myocardial infarction (4), aortic dissection (2), diabetic nephropathy (5), cerebral infarction (2), pancreatic carcinoma (1), hepatocellular carcinoma (3), and gastric cancer (2). The remaining 28 arteries were difficult to excise or used for other purpose.

Coronary arteries were obtained from autopsy cases within 5 to 12 hours after death, and the following examinations were performed within 2 to 5 hours thereafter. A Y-connector was introduced into the proximal portion of the coronary artery being examined for perfusion with saline solution at a rate of 10 ml/min. Then, the angioscope, as described earlier, was introduced through the connector for observation of the artery. Initially, conventional angioscopy was carried out to detect plaque and because the light irradiated from the angioscope tip was visible through the coronary wall, the angioscope tip and, accordingly, the targeted plaque could be confirmed.

After observation by conventional angioscopy, the light guide and the image guide were connected to the fluorescence excitation and emission units, respectively. A band pass filter of 340 ± 15 nm and a band absorption filter of 420 nm were set, and a control image was obtained under perfusion of saline solution. After ceasing the perfusion, 0.5 ml of 2% 3-ANA solution was injected into the perfusion circuit, and 5 minutes later, saline solution perfusion was restarted and the target plaque was imaged again.

A total of 54 plaques were confirmed by conventional angioscopy and CFA in 29 arteries. The 4- to 5-mm-long portion of vessel in which the observed plaque was located was isolated by transecting its proximal and the distal ends at the shorter axes to avoid any damage to the plaque. Subsequently, the isolated segment was cut longitudinally to open the lumen. The 23 normal segments were similarly isolated. The 52 of the 54 isolated coronary segments that contained plaques and 22 of the 23 normal segments were mounted on a deck glass in such a way that the luminal surface of the plaque faced the deck glass. The surface was then scanned by CFM at ×10 or ×40 magnification using

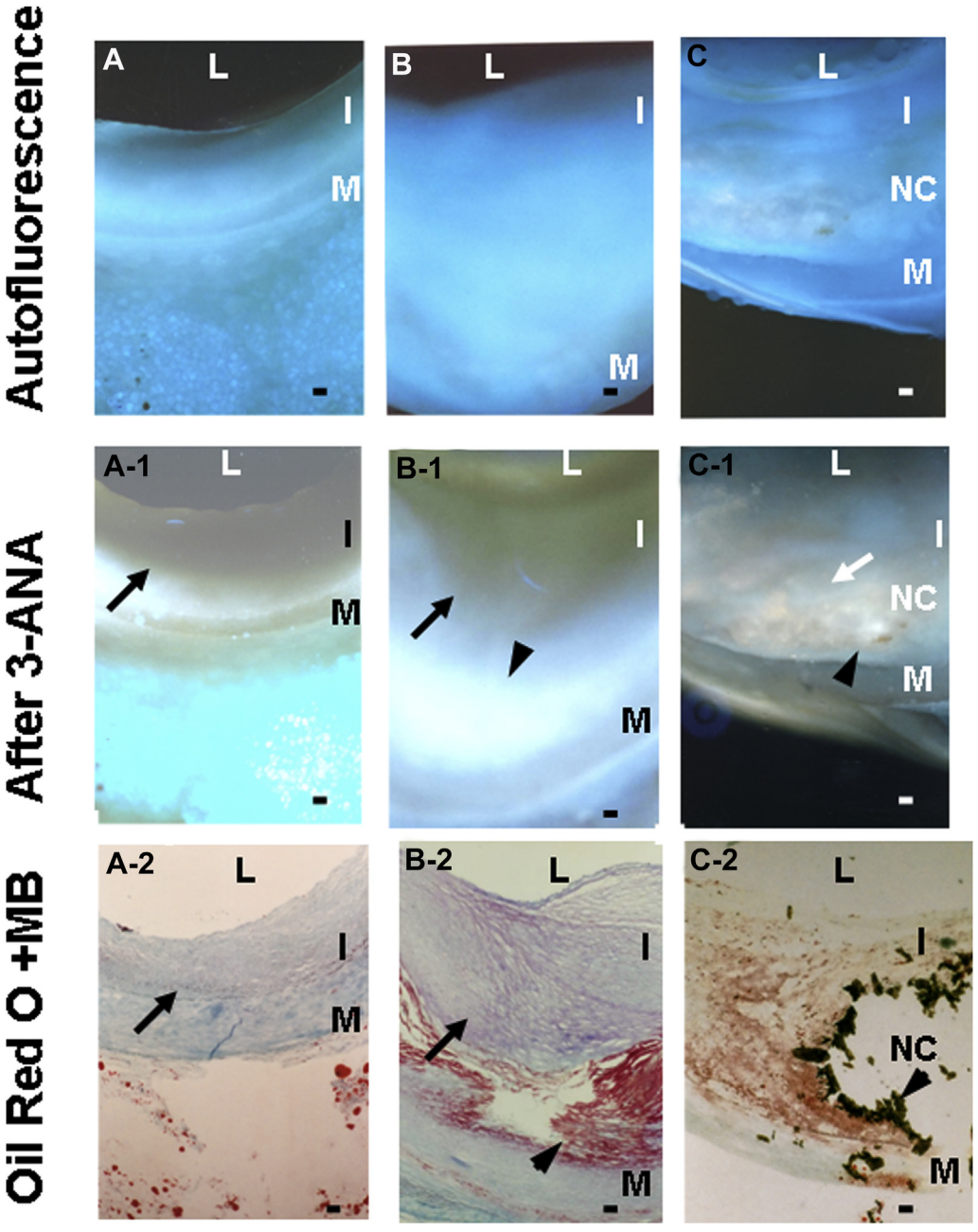

Figure 2. Differences in the deposition sites of TG. *(A–A-2)* Normal coronary segment before the addition of 3-ANA shows a blue fluorescence, indicating the rich presence of collagen I. After the addition of 3-ANA, a brown fluorescence was elicited not only in the intima but also in the media, indicating the presence of TG. *(B–B-2)* White plaque showing TG deposited in the inner layers of the plaque *(arrow in B-1)* but not in the lipid-deposited portions stained red with Oil Red O *(arrowheads in B-1 and B-2)*. *(C–C-2)* Yellow plaque with NC showing TG co-deposited with calcium in a spotty pattern in the NC. *Arrow* in C-1: TG. *Arrowheads* in C-1 and C-2: calcium.

light wavelength filters in similar to those used for CFA. The remaining 2 plaques and 1 normal segment were not used because they were damaged during preparation.

In the 49 plaques and 22 normal segments used for observation of the luminal surface by CFM scanning, the center of the plaque was transected and half was again immersed in 3-ANA solution for 5 minutes because the 3-ANA injected into the perfusion circuit might not have penetrate into the entire wall. Next, it was mounted on a deck glass in such a way that the transected surface faces the deck glass, and the transected surface was scanned by CFM to examine localization of TG. Three plaques were destroyed because of calcium deposition and were not used for scanning. Furthermore, the relations between the deposition site of TG and plaque color determined by conventional angioscopy and the presence or absence of a necrotic core (NC) were examined.

After CFM scanning, the raw sample, which was cut into slices of 30 to 40 μm thickness at the shorter axis, had lipids stained red, calcium as black, collagen fibers and smooth

Table 1
Percentage (%) incidence of triglyceride (TG) deposition in normal segments and plaques of human coronary artery examined by color fluorescent angioscopy (CFA) and microscopy (CFM)

Plaque morphology	Normal segments	White plaques		Yellow plaques	
		Lipid(-)	Lipid (+)	NC(-)	NC(+)
(A) Color fluorescent angioscopy					
n	23	13	16	11	12
TG present	22(95%)	12(92%)	9(56%)	3(27%)	3(25%)*
(B)Color fluorescent microscopy					
(a)Luminal surface scan					
n	22	13	17	11	11
TG present	20 (91%)	10(77%)	8(47%)	3(27%)	2(18%)†
(C) Transected surface scan					
n	22	12	16	10	11
TG present	22(100%)	11(97%)	10(62%)	5(50%)	4(36%)

Normal segments and plaques were classified by conventional angioscopy and histology. The incidence of TG deposition was highest in normal segments, showed a tendency to decrease in white plaques and further in yellow plaques. The incidence in yellow plaques NC (+) was significantly lower than normal segments. No significant difference in incidence was observed between CFA and CFM.

Lipid (−) = lipid absent; Lipid (+) = lipid present; n = number of preparations examined; NC (−) = necrotic core absent; NC (+) = necrotic core present.

* $p < 0.05$ versus normal segments observed by color fluorescent angioscopy.

† $p < 0.05$ versus normal segments observed by color fluorescent microscopic scanning of the luminal surface.

muscles as blue with Oil Red O, and methylene blue dyes for histologic study.

The data obtained were tested by the Fisher's exact test. A value of $p < 0.05$ was considered to be statistically significant.

Results

TG did not autofluoresce, but it presented a brown fluorescence in the presence of 3-ANA. This fluorescent color was not exhibited by any of the other major known substances that comprise atherosclerotic plaques,[2,4] indicating that this fluorescent color was characteristic of just TG.

Figure 1 shows a white plaque by conventional angioscopy. It exhibited blue autofluorescence, indicating the presence of abundant collagen I.[3] After the administration of 3-ANA, the plaque exhibited diffuse brown fluorescence, indicating diffuse deposition of TG. Diffuse brown fluorescence was also observed by luminal surface scanning of the same plaque. On transected surface scanning of the same plaque, brown fluorescence occupied the inner layer (luminal side) of the plaque but not the outer layer (medial side) of the plaque where lipids deposited according to histologic examination.

In the angioscopically normal coronary segments, TG was diffusely deposited in the fibrotic intima in the majority of specimens examined, irrespective of underlying disease or cause of death (Figure 2, Tables 1 and 2), and also frequently in the media (Figure 2). In white plaques without lipid deposition, TG was deposited in the fibrotic intima

Table 2
Deposition sites of triglycerides (TG) examined by transected surface scan of normal segments and plaques by color fluorescent microscopy (CFM)

Plaque morphology	Normal segments	White plaques		Yellow plaques	
		Lipid(-)	Lipid(+)	NC(-)	NC(+)
n	22	12	16	10	11
Deposition sites					
(A) Fibrotic area	22*†‡ (37%)	12§ (100%)	6¶‖ (100%)	2 (20%)	0
(B) Lipid area	0	0	2 (12%)	1 (10%)	1 (9%)
(C) Both areas	0	0 (12%)	2 (20%)	2 (9%)	1
(D) Within NC	0	0	0	0	2 (18%)

Lipid (−) = lipid absent; Lipid (+) = lipid present; n = total number of preparations examined; NC (−) = necrotic core absent; NC (+) = necrotic core present. Both areas = fibrotic and lipid areas.

TG deposited in fibrotic area in normal segments and white plaques Lipid (-) and not only in fibrotic area but also in lipid area in yellow plaques.

* $p < 0.05$ versus yellow plaques NC (−).

† $p < 0.01$ versus yellow plaques NC (+).

‡ $p < 0.0001$ versus lipid area, both area, or within NC.

§ $p < 0.01$ versus lipid area, both area, or within NC.

¶ $p < 0.01$ versus yellow plaques NC (+).

‖ $p < 0.05$ versus within NC.

(Tables 1 and 2), whereas in white plaques with lipid deposition in outer layer (medial side), TG was deposited mostly in the fibrotic area but infrequently in the lipid deposition area (Figures 1 and 2, Table 2). In yellow plaques with or without NC, TG deposition was less frequently observed by CFA and CFM (Tables 1 and 2). In a small number of yellow plaques with NC, TG deposits were observed within the NC (Figure 2, Table 2). As a consequence, the incidence of TG deposition studied by CFA was greatest in the normal segments and showed a tendency to decrease in the order of white plaques without lipid deposition, white plaques with lipid deposition, and yellow plaques without NC. The incidence of TG in yellow plaques with NC that was examined by CFA and CFM luminal surface scan was significantly smaller than that of normal segments (Table 1).

Discussion

Lattermann et al[9] visualized TG in excised rabbit aortic plaques by Raman spectroscopy but not in human coronary plaques. In the present study, a low−molecular weight dye (3-ANA)[1] was found to evoke a brown fluorescence by TG only. Thus, visualization of TG in a given plaque was achieved, enabling analysis of the differences in deposition patterns of TG in human coronary plaques ex vivo. The mechanisms by which 3-ANA evoked fluorescence by TG are not known. One possibility is that 3-ANA became conjugated to TG to form an adduct that provoked the brown fluorescent color. Because there were no other major substances that exhibited a brown fluorescent color, it is believed that it is characteristic of TG and that 3-ANA can be used as a biomarker of TG.

The percentage (%) incidence of TG studied by CFA and CFM was highest in the normal coronary intima in which collagen fibers are abundant and showed to decrease in the

order of white plaques and yellow plaques. In addition, TG was deposited mostly in the fibrotic area (collagen fiber and/or elastic fiber area) but infrequently in the lipid deposition area. That finding suggested that TG deposition decreased with loss of fibrous tissues or with an increase in lipid deposition. It is conceivable that TG was catabolized or replaced by the lipids or was incorporated in the lipoproteins or apolipoproteins. The results in our study are contrary to the general belief that TG content increases with plaque maturation.[10] It is well known that lipoproteins, such as oxidized low-density lipoprotein, low-density lipoprotein, or apolipoprotein B, contain TG.[11,12] These lipoproteins and apolipoproteins increased with plaque maturation in our previous studies in human coronary plaques ex vivo.[2,3] In reported studies, human plaques (mostly carotid plaques excised by endoatherectomy) were homogenized and TG content was measured by biochemical techniques, such as chromatography.[11] We suppose that lipoproteins and apolipoproteins were broken down by homogenization to detach TG; thus, both the detached and pre-existing free TG were measured, showing an apparent increase in TG content in the plaques.

Detection rate by transected surface scan by CFM showed a tendency to be greater than that of CFA. Probably, TG deposited in deeper area was visualized by CFM but not by CFA. Because no significant differences were noted between CFA and CFM in detecting TG within the coronary plaques, the present CFA system has a potential use for imaging TG in the human coronary wall in vivo. If 3-ANA becomes applicable in clinical situation, the relation between plaque TG and ischemic coronary events could be clarified more definitively by the present CFA system in patients in vivo.

The present experimental study entailing the use of 3-ANA as a biomarker of TG appears to be the first to image native TG in the human vascular wall. However, this study has some shortcomings: (1) imaging was limited to the target within 200 μm from the plaque surface, and therefore, deposits in the deeper layers cannot be analyzed by this CFA system.[3] However, by improving the light source, light guide, image guide, and camera, the TG deposited deeper than 200 μm would be visualized. (2) Clinical safety of 3-ANA remains to be clarified before application in patients. (3) The number of patients belonging to each disease group was very small, and therefore, it is not conclusive whether TG deposition in coronary plaques is related to underlying disease.

Disclosures

The authors have no conflicts of interest to disclose.

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